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Central Acetylcholinesterase Reactivation by Oximes Improves Survival and Terminates Seizures Following Nerve Agent Intoxication[1]

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[1]The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

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Abbreviations: ACh = acetylcholine; AChE = acetylcholinesterase; BBB = blood-brain barrier; ChE = cholinesterase; CNS = central nervous system; DAM = diacetylmonooxime; ED_{50} = median effective dose 50%; EEG = electroencephalograph activity; GB = sarin; im = intramuscular; ip = intraperitoneal; LD_{50} = median lethal dose 50%; MINA = monoisonitrosoacetone; MMB-4 = methoxime; OP = organophosphorus compound; 2-PAM = pyridine-2-aldoxime methylchloride; PB = pyridostigmine bromide; RBC = red blood cell; sc = subcutaneous; WB = whole blood.

Abstract

The current treatment regimen for organophosphorus nerve agent intoxication depends on the ability of oximes to rapidly reactivate nerve agent-inhibited acetylcholinesterase (AChE) activity. We have studied the capability of the tertiary oximes monoisonitrosoacetone (MINA) and diacetylmonoxime (DAM), in comparison with the quaternary oximes 2-PAM, HLö7 and MMB-4, to reactivate AChE inhibited by sarin (GB) in blood, brain regions (cortex, hippocampus, striatum, midbrain, cerebellum, brainstem, and spinal cord) and peripheral tissues (heart, diaphragm and skeletal muscle) of guinea pigs. Animals were injected subcutaneously (sc) with 1.0 x LD_{50} of GB and treated intramuscularly (im) five min later with one of the oximes (MMB-4 and HLö7 at 58 µmol/kg, im and 2-PAM, MINA and DAM at 145 µmol/kg, im). Sixty min after nerve agent, blood and tissues were collected and prepared for AChE analysis. All animals survived the 60 min after exposure. AChE reactivation in peripheral tissues and blood was insignificant and in brain regions was significant after treatment with MINA and DAM, whereas AChE reactivation from the quaternary oximes was significant in blood and peripheral tissues, but insignificant in the brain. In another study, animals were pretreated im with pyridostigmine bromide 30 min prior to sc challenge with 2.0 x LD_{50} of GB, and treated im one min later with a combination of atropine sulfate (2.0 mg/kg) and a varied dose of MINA or DAM. With MINA doses of 20, 26, 35, 46 and 60 mg/kg, there were 0, 9, 17, 60, and 75%, respectively, of animals never exhibited EEG seizure activity with 43, 64, 75, 90, and 100%, respectively, survival at 24 hr. With DAM in the dose range from 41 to 231 mg/kg, similar results were obtained. Quaternary oximes did not prevent or stop seizures. These data show that the tertiary oximes reactivated AChE in the brain, improved survival and eliminated or terminated seizures following GB intoxication.

Keywords: Acetylcholinesterase, anticonvulsant, cholinesterase inhibitors, cholinesterase reactivation, diacetylmonooxime, electroencephalogram, guinea pig, monoisonitrosoacetone, nerve agents, organophosphorus compounds, sarin, seizures, survival, tertiary oxime

INTRODUCTION

The potential for exposure to organophosphorus (OP) nerve agents exists on the battlefield, as a terrorist threat to civilian populations, and as an occupational health hazard to workers demilitarizing outdated chemical warfare agents. These agents are extremely potent inhibitors of the cholinesterase (ChE) enzymes, which include acetylcholinesterase (AChE) and butyrylcholinesterase. Their toxic effects are due to hyperactivity of the cholinergic system as a result of inhibition of ChE, in

particular, AChE, and the subsequent increase in the concentration of the neurotransmitter acetylcholine (ACh) in the brain and periphery (Taylor, 2001). Exposure causes a progression of toxic signs, including hypersecretions, muscle fasciculations, tremor, convulsions, respiratory distress, and death (Moore et al., 1995; Taylor, 2001). A combined regimen of prophylaxis and therapy is the most effective medical countermeasure for dealing with the threat of nerve agent poisoning to military personnel (Dunn and Sidell, 1989; Moore et al., 1995; Sidell, 1997; Aas, 2003). Pretreatment with carbamate ChE inhibitors, such as pyridostigmine bromide (PB), shields a fraction of ChE in the periphery from irreversible inhibition by the nerve agents (Berry and Davis, 1970; Dirnhuber et al., 1979). In the event of nerve agent poisoning, immediate therapeutic treatment with an oxime, such as 2-PAM (pralidoxime; pyridine-2-aldoxime methylchloride), obidoxime (toxogonin) or HI-6 (1-(4-carbamoylpyridino) methoxymethyl-2-(hydroxyiminomethyl) pyridinium), is used to reactivate any unaged, inhibited ChE (Wilson and Ginsburg, 1955; Taylor, 2001). Administration of an anticholinergic drug, such as atropine sulfate, antagonizes the effects of excess ACh at muscarinic receptor sites (Moore et al., 1995; Aas, 2003).

Atropine sulfate has been universally adopted as the anticholinergic therapy, but countries vary as to their choice of oxime ChE reactivator. Commercially available oximes for OP poisoning refer to compounds that comprise an oxime moiety (RCH=NOH) attached to a quaternary nitrogen pyridinium ring. They reactivate OP-inhibited ChE by dephosphorylating the enzyme active site via interaction with a nearby anionic subsite. Reactivation occurs through nucleophilic attack by the oxime on the phosphorous atom, splitting an oxime-phosphonate away from the active site. The regenerated esteratic site is subsequently able to bind and cleave its normal substrate, ACh. This action of the oximes is considered to be the major mechanism of their antidotal action in reversing the toxic/lethal effects of nerve agents (Maxwell et al., 2006a, b).

2-PAM chloride is the oxime currently used in the U. S. for the emergency treatment of OP nerve agent exposure. Some countries use different salts (e.g., methanesulfonate, iodide) of 2-PAM. Other countries prefer bis-pyridinium compounds such as obidoxime (toxogonin), trimethoxime (TMB-4) or HI-6 as oxime antidotes (Moore et al., 1995; Kassa, 2002). Although 2-PAM provides adequate protection against the nerve agents sarin and VX (Harris and Stitcher, 1983), it is less effective against other nerve agents (e.g., tabun, soman, cyclosarin) (Fleisher and Harris, 1965; Fleisher et al., 1967). In recent years, several oximes, such as MMB-4 (methoxime; 1,1'-methylenebis[4-[(hydroxyimino)methyl]pyridinium]), HLö7 (1-[[[4-(aminocarbonyl) pyridinio]methoxy]methyl]-2,4-bis[(hydroxyimino)methyl] pyridinium), and HI-6 have been found to possess much better antidotal capacity than 2-PAM in response to nerve agent intoxication in animal studies (Lundy and Shih, 1983; Harris et al., 1989, 1990; Shih et al., 1991; Shih, 1993; Koplovitz and

Stewart, 1994; Dawson, 1994; Kassa, 1998, 2002; Krummer et al., 2002; Maxwell et al., 2008).

Oximes currently used as medical countermeasures have quaternary structures that are very similar, differing only by the number of pyridinium rings and by the position of the oxime moiety on the ring (Figure 1). Quaternary oximes are positively charged compounds, so they cannot cross the blood-brain barrier (BBB), and their action is limited to only the periphery. The inability of quaternary oximes to enter the brain and reactivate nerve agent-inhibited brain AChE is a major limitation of current oxime therapy.

The brain is a major target of OP nerve agents. Inhibition of AChE in the brain results in seizures and neuropathology, thus contributing to the incapacitating and lethal effects of these agents (McDonough and Shih, 1997; Shih et al., 2003). The influence of central AChE on protection is reinforced by the reports showing that partially protecting AChE in the CNS with reversible ChE inhibitors, such as physostigmine, prior to nerve agent exposure could improve survival, reduce seizure activity and neuropathology, and lessen behavioral incapacitation following nerve agent exposure (Fosbraey et al., 1992; Wetherell, 1994; Wetherell et al., 2002). It is, therefore, thought by many medical chemical defense scientists that oxime reactivation of nerve agent-inhibited AChE in the CNS would provide significant benefits. However, there have been no systematic studies to investigate this concept.

Monoisonitrosoacetone (MINA) and diacetylmonooxime (DAM) are two tertiary oximes (see Figure 1) that had been investigated in the 1950's. Both are highly lipid soluble and readily penetrate the BBB (Cohen and Wiersinga, 1960); they are, therefore, able to reactivate AChE within the CNS (Rutland, 1958; Cohen and Wiersinga, 1960). This could have significant impact in the treatment of the toxic effects of OP nerve agents. Indeed, when used alone or in combination with atropine sulfate, MINA and DAM were shown to raise the LD_{50} doses of GB in several animal species (Askew, 1956; 1957; Dultz et al., 1957; Rutland, 1958; Myers, 1959; Wills, 1959). Unfortunately, these two tertiary oximes were not pursued further, due to reports that quaternary pyridinium oximes (e.g., 2-PAM) were more potent reactivators of phosphorylated AChE by several orders of magnitude in human erythrocytes (see review by Hobbiger, 1963).

The present study evaluated the roles of peripheral and central oxime reactivation of nerve agent-inhibited AChE to improve survival and reduce or eliminate other CNS effects of OP nerve agent intoxication in an *in vivo* guinea pig model. To this end two studies were performed. A reactivation experiment compared the tertiary oximes MINA and DAM with several quaternary oximes, 2-PAM, MMB-4 and HLö7 in their ability to reactivate GB-inhibited AChE activity in blood, peripheral tissues and brain regions. An anticonvulsant experiment compared the capacity of these two tertiary oximes with the quaternary oximes for their ability to prevent/terminate GB-induced seizures and improve survival.

MATERIALS AND METHODS

Subjects. Male Hartley guinea pigs (Crl:(HA) BR COBS) weighing 250-300 g were purchased from Charles River Labs (Kingston, NY). They were housed in individual cages in temperature (21 ± 2°C) and humidity (50 ± 10%) controlled quarters that were maintained on a 12-h light – dark schedule (with lights on at 0600 h). Laboratory chow and tap water were freely available whenever the animals were in home cages. Animals were quarantined for one week prior to experimentation. The research environment and protocols for animal experimentation were approved by the Institutional Animal Care and Use Committee (IACUC). In conducting the research described in this report, the investigators adhered to the Guide for the Care and Use of Laboratory Animals by the Institute of Laboratory Animal Resources, National Research Council, in accordance with the stipulation mandated for an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) accredited facility.

Materials. Saline (0.9% NaCl) injection, USP, was purchased from Cutter Labs, Inc. (Berkeley, CA). MINA (Monoisonitrosoacetone; anti-pyruvic aldehyde 1-oxime, 98% pure), DAM (diacetylmonooxime; 2,3-butanedione monoxime, ≥98% pure), acetylthiocholine iodide, and atropine sulfate were purchased from Sigma-Aldrich (St. Louis, MO). Pyridostigmine bromide (PB) was obtained from Hoffmann-La Roche, Inc. (Nutley, NJ), and pyridine-2-aldoxime methylchloride (2-PAM) was purchased from Ayerst Labs, Inc. (New York, NY). HLö7 (1-[[[4-(aminocarbonyl)pyridinio]methoxy] methyl]-2,4-bis[(hydroxyimino)methyl] pyridinium dimethanesulfonate) and MMB-4 (methoxime; 1,1'-methylene bis [4-[(hydroxyimino)methyl]pyridinium] dimethansulfonate) were obtained from the depository at Walter Reed Army Institute of Research (Washington, DC). Bicinchoninic acid (BCA) Reagent A (sodium carbonate, sodium bicarbonate, BCA and sodium tartrate in 0.1 M sodium hydroxide), BCA Reagent B (4% cupric sulfate), and 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) were purchased from Pierce Biotechnology, Inc. (Rockford, Illinois). DTNB was prepared in Tris buffer (0.05 M, pH 8.2) to a concentration of 0.424 M. Attane™ (Isoflurane, USP) was purchased from Minrad, Inc. (Bethlehem, PA). Buprenorphine HCl was purchased from Reckitt Benckiser Pharmaceuticals, Inc. (Richmond, VA). Heparin sodium was purchased from U.S.P., Inc. (Rockville, MD). The OP chemical warfare nerve agent studied was sarin (GB; isopropylmethylphosphono fluoridate). Sarin was obtained from the US Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD). Nerve agent was diluted in ice-cold saline prior to subcutaneous (sc) injection. PB, atropine sulfate, and oxime compounds were prepared in saline for intramuscular (im) injection. Injection volumes were 0.5 ml/kg for nerve agent and all treatment drugs.

Reactivation Experiment. One to 3 days prior to the experiment, about 0.5 ml blood was drawn (Vallejo-Freire, 1951) and collected into a 1.0-ml microfuge tube

containing 50 µl of heparin sodium (15 units/ml) to determine baseline AChE activity in whole blood (WB) and red blood cells (RBC). On the day of the study, groups of guinea pigs were injected subcutaneously (sc) with either saline (0.5 ml/kg) or a 1.0 x LD_{50} dose of GB (42.0 µg/kg). Five min later, when the inhibition of blood AChE activity by GB reached maximum (Shih et al., 2005), saline (0.5 ml/kg), HLö7 (30.2 mg/kg), MMB-4 (26.0 mg/kg), 2-PAM (25.0 mg/kg), MINA (12.63 mg/kg), or DAM (14.66 mg/kg) was given intramuscularly (im). No atropine sulfate therapy was given. Control animals received sc saline (no nerve agents) and im saline (no oximes). There were 8 animals assigned to each treatment group. Additionally, dose-response effects of MINA and DAM alone or following GB exposure on AChE activity were also investigated.

Sixty min after sc saline or GB administration, the animals were deeply anesthetized with isoflurane and euthanized by decapitation. Shortly before anesthesia, the severity of toxic signs of each animal was scored (see below). Blood (~0.5 ml) was collected into a 1.0-ml microfuge tube containing 50 µl of heparin sodium solution (15 U/ml). For the WB samples, 20 µl of blood was diluted 1:25 in 1% Triton–X100 solution. For the RBC samples, the original blood sample was centrifuged for 5 min at 14,000 rpm, and 10 µl of the RBC was then diluted 1:50 in 1% Triton–X100 solution. Brain regions (brainstem, cerebellum, cortex, hippocampus, midbrain, spinal cord and striatum) and peripheral tissue (diaphragm, heart, and skeletal muscle) were dissected. Brain samples were diluted 1:20, while peripheral samples were diluted 1:5, in 1% Triton-X 100 solution (in water) and then homogenized. The homogenates were then centrifuged (31,000 x g at 4^{o}C; 20 min for brain and 30 min for peripheral tissues) and the supernatant decanted and kept frozen at -80^{o}C until analysis (Shih et al., 2005).

Treatment Dose Rationale. The 25-mg/kg dose of 2-PAM approximates the total dose of 2-PAM in 3 autoinjectors (600 mg per injector) given as immediate nerve agent treatment to a 70- to 75-kg human. The 25-mg/kg dose of 2-PAM is equivalent to a 145 µmol/kg dose, and the initial MINA and DAM doses (12.63 and 14.66 mg/kg, respectively) were matched to this 145 µmol/kg dose (molecular weight of MINA = 87.08 and of DAM = 101.10). Later, additional doses of MINA (17.5, 35, 60, or 80 mg/kg, im) and DAM (23, 41, 73, or 128.8 mg/kg, im) were added and examined. Both MMB-4 and HLö-7 are bis-pyridinium compounds similar to HI-6; a 58-µmol/kg dose was used for MMB-4 and HLö7, based on 3-autoinjector equivalent dose (500 mg per injector) of HI-6 (Clair et al., 2000).

Toxic Signs Test. At 58 min after GB injection, guinea pigs were observed for signs of cholinergic toxicity (Table 1), including secretions (salivation or lacrimation), motor deficits, and general state (activity and coordination). This toxic sign score system for guinea pigs in anti-AChE agent-treated animals was modified from that of Shih and Romano (1988) reported for rats. They were scored for absence [0] or presence [1] of each of the following signs: salivation, lacrimation, and nystagmus. General motor signs were assessed a 0-3 score: normal = 0, fasciculation = 1, tremor

= 2, or convulsion = 3. Next, the guinea pig was allowed to walk on the bench top and general state was assessed a 0-3 score: normal = 0, mild uncoordination = 1, impaired movement/with righting reflex = 2, or prostration/no righting reflex = 3. A cumulative score was then calculated by tabulating the salivation, lacrimation, nystagmus, general motor and general state score for each subject. The maximal attainable score was 9 (Table 1). A cumulative score was categorized as mild intoxication [1-3], moderate intoxication [4-6] and severe intoxication [7-9].

AChE Analysis. The AChE activity was measured spectrophotometrically using a variation of the microplate method modified from Ellman et al. (1961), and a BCA protein assay was used to obtain protein concentrations in the tissue samples to standardize AChE levels among tissues (Shih et al., 2005). On the day of AChE analysis, the samples were thawed, and three 7-µl replicates of each tissue sample and three 10-µl replicates of the WB and RBC samples were pipetted into a 96-well UV star microplate (Greiner, Longwood, FL). Twenty µl of deionized water was added to each well containing brain and peripheral tissue samples, and 17 µl of deionized water was added to each WB and RBC sample. Then 200 µl of DTNB (0.424 M, pH 8.2) was added as the chromatophore to each sample well. Each microplate was then incubated for 10 min at 37^{o}C before being placed in the Spectramax Plus microplate reader (Molecular Devices, Sunnyvale, CA) where it was allowed to shake for 2 min. Immediately after, 30 µl of the substrate acetylthiocholine iodide (51.4 mM) was added to each well. The samples were read at 410 nm (at 20-sec intervals) for 2.0 min (tissues) or 3.5 min (blood), and the activity (µmol substrate hydrolyzed/ml/min) was determined using Softmax plus 4.3 LS software (Molecular Devices).

Protein Analysis. Protein levels in the tissue samples were determined by a BCA protein assay. The standard curve was created using bovine serum albumin at the following concentrations: 0.5, 0.75, 1.0, 1.5 and 2.0 mg/ml. Three replicates of 10 µl for each brain tissue sample and three replicates of 5 µl for each peripheral tissue sample were added to individual microplate wells. The peripheral tissue samples were further diluted by adding 5 µl of deionized water. Then 200 µl of BCA working reagent was added to each well. The microplates were shaken for 30 sec and then incubated at 37^{o}C for 30 min. The microplates were allowed to cool to room temperature before being read at 562 nm (single read) using the Spectramax Plus microplate reader and Softmax Plus 4.3 LS software as described above. After obtaining the protein contents of each tissue sample, the AChE activity is then expressed as µmol substrate hydrolyzed/g protein/min for brain and peripheral tissues.

Anticonvulsant Experiment. Approximately one week before experimentation the animals were implanted with stainless-steel cortical screw electrodes to record electroencephlographic (EEG) signals. The animals were given an initial dose of buprenorphine HCl (0.03 mg/kg, sc) and anesthetized with isoflurane (3% induction, 1.5-2% maintenance; with oxygen) and set in a stereotaxic frame. Three cortical

stainless-steel screw electrodes were implanted in the skull: two were placed bilaterally ~3.0 mm lateral from the midline and equidistant between bregma and lambda; the third was placed on the posterior calvaria as the reference electrode. Stainless-steel wires attached the screws to a miniature connector plug. The electrodes, wires and plug were encased in cranioplastic cement. The incision was sutured; the animal was removed from the frame, given buprenorphine HCl (0.03 mg/kg, sc) for postoperative analgesia and placed on a warming pad for at least 30 min after recovery before being returned to the animal quarters (Shih and McDonough, 1999; Shih et al., 2003; 2007).

On the day of study, the animals were placed in recording chambers and at least a 15-min baseline EEG was recorded. EEG activities were recorded using CDE 1902 amplifiers and displayed on a computer running Spike2 software (Cambridge Electronic Design, Ltd., Cambridge, UK). After a 15-min recording of baseline EEG activity, animals received a dose of PB (0.026 mg/kg, im) to produce a 20-30% WB AChE inhibition (Lennox et al., 1985). Thirty min later, animals were challenged with 2 x LD_{50} (sc) GB. One min after nerve agent challenge, animals were treated im with atropine sulfate (2.0 mg/kg) plus HLö7 (30.2 mg/kg), MMB-4 (26.0 mg/kg), 2-PAM (25 mg/kg), MINA (26 – 60 mg/kg), or DAM (82 – 231 mg/kg). The method of probit analysis (Bliss, 1952), using 4-7 doses with 5-6 animals per group, was used to establish an estimated anticonvulsant ED_{50} with 95% fiducial limits for each oxime against GB.

Animals were observed continuously for the first hour following exposure and treatment, and periodically thereafter for at least 6 hr. EEG activity was recorded continuously throughout this time and, if the animal survived, for another 30 min at 24 hr after exposure. Seizure onset was operationally defined as the appearance of ≥ 10 sec of rhythmic high amplitude spikes or sharp wave activity in the EEG tracing. Each animal was rated as never having a seizure or having the seizure terminated (OFF) or not terminated (NOT OFF) based on the overall appearance of the EEG record at the end of the experimental day and during the 24-hr observation. (Note: An animal was rated as OFF if a seizure never occurred or if the seizure terminated and the EEG remained normal at all subsequent observation times.) Animals rated as NOT OFF had obvious epileptiform activity that never stopped in response to treatment or reappeared in the EEG record before the end of the 6-hr recording on the day of exposure and/or during the 30-min recording at the 24-hr observation time. Mortality and body weight of survivors were recorded 24 hr after nerve agent exposure. Body weight changes are an indicator of long-term health and survivor following nerve agent exposure (McDonough et al., 1989; 1998; Shih et al., 1990). Animals that survived 24 hr were euthanized with an overdose of sodium pentobarbital (75 mg/kg, ip) and then perfused through the aorta with saline followed by 10% neutral-buffered formalin for later pathological evaluations (data not presented).

Data analysis. AChE activity was expressed as μmol substrate hydrolyzed/ml/min for blood samples and as μmol substrate hydrolyzed/g protein/min for brain and

peripheral tissue samples. The enzymatic activities of the treatment groups were then expressed as percentage of the saline-saline control group. Statistical analysis of enzymatic activities and bodyweight changes were performed using one-way ANOVAs to compare across treatments. A post hoc Tukey test was used for multiple comparisons. In cases where equal variances could not be assumed, a Dunnett C post hoc test was used. Differences in incidence of toxic signs between treatment groups were evaluated using Fisher's Exact Test. Statistical significance was defined as $p<0.05$. Dose-effect curves and the median effective doses (ED_{50}) for anticonvulsant activity of oxime were determined by probit analysis (Bliss, 1952) using 4-7 doses with 5-6 animals per group. A probit regression analysis (SPSS for Windows, Version 14.0, Chicago, IL) was used to estimate the ED_{50} values along with the 95% confidence intervals for each oxime treatment.

RESULTS

Reactivation Experiment

Signs of toxicity and lethality. Under the conditions of this study, guinea pigs exposed to a 1.0 x LD_{50} (42 µg/kg, sc) of GB and not receiving any therapy exhibited signs of nerve agent intoxication, such as salivation, rhinorrhea, tremor, muscle fasciculations, and convulsions. Table 2 and Figure 2 display these data. The animals that received 1.0 x LD_{50} of GB followed by saline (saline-treated controls) showed a high incidence (93%; 14 of 15 animals) of toxic signs, and the average total scores (4.07) were in the moderate range. Within 60 min three out of ten of these saline-treated controls had died. Animals treated with 2-PAM (145 µmol/kg), MMB-4 (58 µmol/kg) or HLö-7 (58 µmol/kg) all showed a similar incidence (100%) of toxic signs as did the saline controls, but were rated in the mild to moderate range (from 2.75 to 4.13). The numbers of animals showing toxic signs of nerve agent intoxication were significantly less for those treated with MINA (26%, 10 of 39) or DAM (20%, 8 of 40; $p< 0.001$; Fisher's Exact Test) than the saline-treated controls, and the severity of toxic signs were all mild (range from 0.00 to 0.88). No animal treated with any quaternary or tertiary oxime died within 60 min of GB exposure.

AChE activity in brain regions. Basal AChE activities in the brainstem, cerebellum, cortex, hippocampus, midbrain, spinal cord and striatum are shown in Table 3 (top row). The AChE activity in striatum was significantly higher than any other region. The next highest AChE activity was found in the cerebellum and brainstem, followed by spinal cord and midbrain, which were significantly higher than hippocampus. Cortex had the lowest AChE activity among all brain regions, approximately one sixth of that of striatum.

Effects of GB alone. The ability of GB to inhibit brain regional AChE activity is shown in Table 3 (bottom row). Sixty min following exposure to a 1.0 x LD_{50} dose of GB, AChE activities in the brainstem, cerebellum, cortex, hippocampus, midbrain,

spinal cord and striatum were inhibited to 19, 11, 10, 24, 14, 30, and 20% of control, respectively. The rank order of AChE inhibition by GB in brain regions (from high to low) was cortex = cerebellum = midbrain > brainstem = striatum > hippocampus = spinal cord.

Effects of oximes. The doses of MINA, DAM, 2-PAM, MMB-4 or HLö7 by themselves alone did not affect (inhibit or potentiate) AChE activity in any brain regions (data not shown). The results of oxime's ability to reactivate sarin-inhibited AChE activity are shown in Table 4. None of the quaternary oximes (2-PAM at 145 µmol/kg; MMB-4 and HLö7 at 58 µmol/kg) reactivated GB-inhibited AChE activity in any brain regions. The two tertiary oximes (MINA and DAM at 145 µmol/kg) reactivated GB-inhibited AChE in the brain and spinal cord with regional specificity. MINA reactivated (i.e., had significantly higher AChE activity when compared with GB-exposed saline-treated group) AChE activity inhibited by GB in brainstem, midbrain, and striatum, while DAM reactivated AChE in cerebellum, midbrain, spinal cord, and striatum. Figure 3 shows the significant AChE reactivation effects observed in two brain regions: striatum and midbrain.

As the doses of MINA or DAM were increased, a dose-dependent AChE reactivating activity was observed for MINA as seen in Figure 4. DAM, on the other hand, showed dose-dependent AChE reactivation from 14.66 to 41 mg/kg, reaching a plateau at 41 mg/kg. With higher doses of DAM (73 and 128.8 mg/kg), there was a trend of reducing AChE activity, although those AChE activities were still significantly higher than in GB-exposed saline-treated control animals. Figure 4 displays the dose-related AChE reactivating effects of MINA and DAM.

AChE activity in peripheral tissues and blood. Basal AChE activities in diaphragm, heart and skeletal muscle are shown in Table 5 (top row). The rank order from high to low of control AChE in the 3 peripheral tissues is heart > diaphragm > skeletal muscle, while in the RBC and WB the AChE activity was about equal.

Effects of GB alone. The ability of GB to inhibit peripheral tissue and blood AChE activity is shown in Table 5 (bottom row). Sixty min following exposure to a 1.0 x LD_{50} dose of GB, AChE activities in the diaphragm, heart, and skeletal muscle were inhibited to about 28, 17 and 40% of control, respectively, and in RBC and WB to about 7 and 9% of control, respectively. The rank order of AChE inhibition by GB from high to low was heart > diaphragm > skeletal muscle. In RBC and WB, the degree of AChE inhibition by GB was similar.

Effects of oximes. The doses of MINA, DAM, 2-PAM, MMB-4 or HLö7 by themselves alone did not affect (inhibit or potentiate) AChE activity in blood and any peripheral tissues. The AChE reactivating results are shown in Table 6 and Figures 5-7. In the peripheral tissues and blood, both tertiary oximes at the 145 µmol/kg dose (MINA = 12.63 mg/kg and DAM = 14.66 mg/kg) was not able to reactivate GB-inhibited AChE (displayed in Figures 5 and 7); however, as shown in Figures 6 and 8 both were able to do so at higher doses (e.g., MINA at 60 or 80 mg/kg and DAM at 41 mg/kg). All three quaternary oximes (HLö7, MMB-4, or 2-PAM), on

the other hand, were readily able to reactivate GB-inhibited AChE in peripheral tissues and in blood. Figures 5 and 7 show the AChE reactivating effects of equimolar dose of oximes following 1.0 x LD_{50} GB exposure in the peripheral tissues and blood, respectively. In the diaphragm and skeletal muscle (Figure 5), HLö7, MMB-4 and 2-PAM markedly reactivated GB-inhibited AChE, with HLö7 and MMB-4 having significantly greater AChE reactivation than 2-PAM. In the heart, HLö7, MMB-4 and 2-PAM significantly reactivated AChE activity to a similar degree when compared with the GB-exposed saline-treated control. In the RBC (Figure 7), MMB-4 reactivated significantly more AChE activity inhibited by GB than did HLö7 and 2-PAM, while in the whole blood, HLö7, MMB-4 and 2-PAM significantly reactivated AChE activity to a similar degree when compared with the GB-exposed saline-treated control.

Anticonvulsant Experiment

Seizure Occurrence and Survival at 24 hr. Table 7 shows the incidence of EEG seizure occurrence and 24-hr survival. In guinea pigs exposed to 2.0 x LD_{50} GB (84 µg/kg, sc) and treated one min later with atropine sulfate (2.0 mg/kg, im) plus any quaternary oxime (2-PAM, MMB-4 or HLö7), all animals (100%) developed continuous seizure activity (*status epilepticus*) and only 20 - 50% of animals survived 24 hr. With MINA at doses of 20, 26, 35, 46 and 60 mg/kg, 0, 9, 17, 60, and 75% of animals, respectively, never exhibited EEG seizure activity and 43, 64, 75, 90, and 100% of these animals, respectively, survived 24 hr. Similarly, with DAM at doses of 41, 73, 129 and 231 mg/kg, 0, 17, 67 and 100%, respectively, of animals never exhibited EEG seizure activity and 71, 83, 100 and 100% of these animals, respectively, survived 24 hr.

Seizure Onset. The seizure onset times for 2-PAM-, MMB-4- and HLö7-treated animals were 6.1$\pm$0.4 (n=10), 6.4$\pm$0.4 (n=5), and 6.7$\pm$1.1 (n=5) min after GB exposure, respectively. In those animals that displayed EEG seizure activity, seizure onset times after 2 x LD_{50} of GB are 7.4$\pm$0.4 (n=33) min for MINA- and 7.0$\pm$0.8 (n=14) min for DAM-treated groups. There was no significant difference in time to seizure onset among these five oxime groups.

Seizure Termination. In animals treated with atropine sulfate (2.0 mg/kg, im) plus 2-PAM, MMB-4 or HLö7 at one min after GB (2.0 x LD_{50}), the EEG seizure activity induced by GB never abated, although at 24 hr the amplitude and frequency of spiking activity were significantly reduced. Figure 9 shows a typical EEG tracing of the ineffectiveness of a quaternary oxime (i.e., MMB-4) to terminate GB-induced seizure activity. In animals treated with MINA at doses that were sufficient to terminate EEG seizure activity, the average termination time was 5.2 min. Figure 10 displays the GB-induced EEG seizure activities (seizure onset at 3.9 min) that were terminated by MINA (35 mg/kg) at 7.5 min after GB administration. Similarly, animals treated with the various doses of DAM either failed to develop seizures or, if seizures did develop, they were continuous throughout the recording period as was seen with the quaternary oximes. Figure 11 shows a typical GB-induced EEG

seizure (seizure onset at 9.5 min) that could not be overcome by DAM at 41 mg/kg, im; note the continued EEG spiking even at 24 hr after treatment. In addition, animals treated with 2-PAM, MMB-4 or HLö7 experienced weight loss (of 30% of pre-exposure body weight) that was equivalent to that shown by the GB-exposed saline-treated controls. Those animals treated with MINA or DAM, but with seizures terminated, experienced significantly less ($p < 0.001$) body weight loss over the 24-hr survival period than did animals with seizures not terminated and animals treated with 2-PAM, MMB-4 or HLö7. These data are displayed in Figure 12.

Anticonvulsant Efficacy. Figure 13 shows the anticonvulsant dose-response effects of MINA and DAM in our anticonvulsant test model. The anticonvulsant ED_{50} (with 95% confidence intervals) for MINA in the presence of atropine sulfate (2.0 mg/kg) against 2.0 x LD_{50} of GB was 36.65 (0.00 – 76.30) mg/kg, im, whereas the anticonvulsant ED_{50} for DAM was determined to be 112.51 (83.78 – 178.87) mg/kg, im. Thus, the anticonvulsant efficacy for MINA was significantly more potent than that for DAM under similar conditions.

DISCUSSION

In the present study we investigated the AChE reactivating ability and anticonvulsant capacities of two tertiary oximes, MINA and DAM, and compared their effectiveness with three quaternary oximes, 2-PAM, HLö7 and MMB-4, in GB-exposed guinea pigs. In the reactivation study, animals were challenged with a 1.0 x LD_{50} dose of GB followed 5 min later by treatment with saline (served as control) or a selected dose of an oxime. CNS and peripheral tissues and blood were collected at 60 min after GB exposure for AChE activity determination. Both quaternary and tertiary oximes were able to reactivate GB-inhibited AChE, but in an opposite direction with respect to tissue specificity. In the CNS only the tertiary oximes reactivated GB-inhibited AChE, whereas in the blood and peripheral tissues all three quaternary oximes (and only higher doses of MINA and DAM) reactivated GB-inhibited AChE. In the anticonvulsant study, guinea pigs were pretreated with PB to inhibit ~20-30% of blood AChE activity 30 min prior to challenge with a 2.0 x LD_{50} of GB. One min later an injection of atropine sulfate (2.0 mg/kg, im) and a dose of oxime were administrated. The EEG activity and mortality rate were followed for up to 6 hr and again at 24 hr after GB exposure. Under these conditions, both MINA and DAM, but none of the 3 quaternary oximes, prevented or quickly stopped seizure activity, with MINA displaying a higher potency than DAM. At higher MINA or DAM doses, many animals never developed EEG seizure activity and lost only a modest amount of body weight over the 24-hr survival period.

In the AChE reactivation study, we chose a 145-μmol/kg dose of 2-PAM, which is equivalent to 3 auto-injectors of the Mark I nerve agent antidote kit for a 70- to 75-kg human. Because the molecular weights of both MINA and DAM are

quite low, we also used 145 µmol/kg for these two tertiary oximes. On the other hand, since both MMB-4 and HLö7 are bis-pyridinium compounds similar to HI-6, a 58-µmol/kg dose was used for MMB-4 (26 mg/kg) and HLö7 (30.2 mg/kg), based on a 3-autoinjector equivalent dose (500 mg per injector) of HI-6 (Clair et al., 2000). With the dose of 145 µmol/kg, neither MINA (12.63 mg/kg) nor DAM (14.66 mg/kg) was able to reactivate GB-inhibited AChE in blood (RBC and WB) and peripheral tissues (diaphragm, heart and skeletal muscle). The AChE reactivation in blood and peripheral tissues was observed only at much higher doses of MINA (60 or 80 mg/kg) and DAM (41 mg/kg, but not 73 or 128.8 mg/kg). The reason for this effect of DAM is not clear, but is probably due to the reported observation that DAM binds initially to carboxylesterase in the plasma of guinea pigs after absorption and reactivates OP-inhibited carboxylesterase, thus reducing its availability to bind and reactivate AChE (Myers, 1959). On the other hand, 2-PAM (at 145 µmol/kg), MMB-4 and HLö7 (both at 58 µmol/kg) reactivated GB-inhibited AChE in blood and peripheral tissues quite well, with both MMB-4 and HLö7 showing a higher reactivating rate than that of 2-PAM.

As can be expected 2-PAM, MMB-4 and HLö7 did not show any AChE reactivation in the CNS, since they do not penetrate the BBB due to their quaternary structure and limited lipid solubility. On the other hand, MINA and DAM are tertiary structures and highly lipid soluble and, at higher doses, significantly reactivated GB-inhibited AChE in most of the brain regions (including spinal cord) studied. At a dose of 145 µmol/kg, MINA reactivated AChE activity significantly in brainstem, midbrain, and striatum, while DAM significantly reactivated the AChE activity in cerebellum, midbrain, spinal cord, and striatum. The differences in the regional specificity of these two tertiary oximes are not understood, but may be due to their individual distribution profile in brain regions. However, at higher doses the AChE reactivating capacity in the CNS was highly significant for both MINA and DAM, with MINA being more potent than DAM.

Another noteworthy observation in the reactivation study was that, while MINA produced a dose-dependent increase in brain regional AChE reactivation from 12.6 to 80 mg/kg, the reactivation profile of DAM reached a plateau at 41 mg/kg in all tissues. From the 73 to 128.8 mg/kg doses of DAM, there were no further increases of the AChE activities, but the activity were appeared to be reduced, although the overall AChE activity was still significantly higher than those inhibited by GB. This phenomenon produced by DAM, unlike MINA, in the CNS is not clear. Even though the CNS AChE reactivation produced by DAM was not quite dose-dependent beyond 41 mg/kg, animals treated with increasingly higher doses of both MINA and DAM clearly displayed less toxic signs of GB-induced cholinergic hyperactivity than observed in those animals treated with 2-PAM, MMB-4 or HLö7. These observations were confirmed by the results of our anticonvulsant study.

In the anticonvulsant study, both MINA and DAM produced anticonvulsant effects with MINA having greater potency than DAM. When MINA or DAM was

administered along with 2.0 mg/kg atropine sulfate in PB-pretreated guinea pigs, EEG seizure activities induced by 2.0 x LD_{50} of GB were prevented or quickly arrested. As the doses of MINA or DAM were increased, greater percentages of animals never exhibited EEG seizure activity. If seizure activity did occur, it was spontaneously terminated within minutes (averaged 5.2 min). Thus, increasing the doses of MINA or DAM reduced seizure occurrence and increased the propensity for seizure termination. Additionally, it was observed that increases in the dose of MINA or DAM enhanced survival and minimized 24-hr weight loss. On the other hand, the quaternary oximes, 2-PAM, MMB-4, and HLö7, had no effect on GB-induced seizure activity and, while preventing lethality, did not protect against the GB-induced body weight loss of about 30%.

Both MINA and DAM had been investigated as potential oximes for the treatment of nerve agent (i.e., GB) exposure in the 1950's (Askew, 1956; 1957; Dultz et al., 1957; Rutland, 1958; Myers, 1959; Wills, 1959). In view of current pharmacological data, it was unfortunate that these two tertiary oximes were not pursued further because of the reports that quaternary pyridinium oximes were several orders of magnitude better reactivators of phosphrylated AChE in human erythrocytes (see review by Hobbiger, 1963). Both are lipid soluble and can readily penetrate the BBB (Cohen and Wiersinga, 1960), as was confirmed with our reactivation study, and this CNS AChE reactivation has significantly beneficial functional consequences as shown with the current anticonvulsant study. When MINA or DAM was administered at high doses one min after 2.0 x LD_{50} of GB, many of the animals never expressed EEG seizure activity, or the bursting seizure activity could be quickly stopped. These tertiary oximes are able to reactivate AChE within the CNS (Rutland, 1958; Cohen and Wiersinga, 1960). Our present data not only supported these earlier findings, but further showed that effective reactivation of AChE in the brain can increase survival and prevent seizure and possibly associated neuropathology. Askew (1956, 1957) showed in the late 1950's that when used alone or in combination with atropine sulfate, MINA and DAM markedly raised the LD_{50} doses of GB in mice, rats, guinea pigs, and rabbits. In our anticonvulsant study, we confirmed that both MINA and DAM increased survival in guinea pigs. We also showed that both MINA and DAM possessed anticonvulsant effectiveness against GB, with MINA being more potent in this respect. It has been our observations (McDonough and Shih, 1997; McDonough et al., 1999, 2000; Shih et al., 2003; 2007) that if nerve agent-induced seizure activity can be eliminated rapidly with any number of different effective anticonvulsant treatment drugs, there will be less brain pathology and more probability of survival. It is reasonable to predict that with MINA or DAM included in a therapeutic regimen for OP nerve agent poisoning, significantly less brain pathology and associated behavioral abnormalities might be seen in survivors.

In conclusion, this study clearly shows that tertiary oximes (DAM and MINA) reactivated AChE in the brain, reduced toxic signs, improved survival, and prevented

or spontaneously terminated seizures following GB intoxication. The current results support the notion that central AChE reactivation or preservation of CNS AChE activity following nerve agent intoxication is critical in the medical management of nerve agent intoxication (Fosbraey et al., 1992; Wetherell, 1994; Wetherell et al., 2002). Thus, tertiary oximes with high lipid solubility could be an excellent adjunct to current pretreatment and therapy regimens for medical management of OP nerve agent intoxication.

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FIGURE LEGENDS

Figure 1. Chemical structures of quaternary and tertiary oximes studied.

Figure 2. The overall toxic sign scores in animals exposed to a 1.0 x LD_{50} dose of sarin and treated 5 min later with saline (served as control), quaternary (2-PAM, MMB-4 and HLö7) or tertiary (DAM and MINA) oximes. Toxic signs were scored at 58 min after sarin administration in survivors.
[*] $P<0.05$ compared with saline control group.
[#] $P<0.05$ compared with quaternary oxime group.

Figure 3. AChE activity in striatum and midbrain regions of the guinea pigs exposed to sarin (1.0 x LD_{50}, sc) and treated intramuscularly 5 min later with saline (as control), 58 µmol/kg of HLö7 and MMB-4, or 145 µmol/kg of 2-PAM, MINA and DAM. Brain tissues were collected at 60 min after sarin exposure. AChE activities were expressed as mean % baseline activity $\pm$ SEM with N= 8 in each treatment group.
[#] $p<0.05$ compared with agent/saline group (control).
[○] $p<0.05$ compared with HLö7 group.
[□] $p<0.05$ compared with MMB-4 group.
[*] $p<0.05$ compared with 2-PAM group.

Figure 4. AChE activity in striatum and midbrain regions of the guinea pigs exposed to sarin (1.0 x LD_{50}, sc) and treated intramuscularly 5 min later with saline (as control) or various doses of MINA or DAM. Brain tissues were collected at 60 min after sarin exposure. AChE activities were expressed as mean % baseline activity $\pm$ SEM with N= 8 in each treatment group.
[#] $p<0.05$ compared with agent/saline group (control).
[○] $p<0.05$ compared with HLö7 group.
[□] $p<0.05$ compared with MMB-4 group.
[*] $p<0.05$ compared with 2-PAM group.

Figure 5. AChE activity in the diaphragm, heart and skeletal muscle of the guinea pigs exposed to sarin (1.0 x LD_{50}, sc) and treated intramuscularly 5 min later with saline (as control), 58 µmol/kg of HLö7 and MMB-4, or 145 µmol/kg of 2-PAM, MINA and DAM. Tissues were collected at 60 min after sarin exposure. AChE activities were expressed as mean % baseline activity $\pm$ SEM with N= 8 in each treatment group.
[#] $p<0.05$ compared with agent/saline group (control).
[○] $p<0.05$ compared with HLö7 group.
[□] $p<0.05$ compared with MMB-4 group.
[*] $p<0.05$ compared with 2-PAM group.

Figure 6. AChE activity in the diaphragm, heart and skeletal muscle of the guinea pigs exposed to sarin (1.0 x LD_{50}, sc) and treated intramuscularly 5 min later with saline (as control), or various doses of MINA or DAM. Tissues were collected at 60 min after sarin exposure. AChE activities were expressed as mean % baseline activity $\pm$ SEM with N= 8 in each treatment group.
$^{\#}$ $p<0.05$ compared with agent/saline group (control).
$^{\circ}$ $p<0.05$ compared with HLö7 group.
$^{\square}$ $p<0.05$ compared with MMB-4 group.
* $p<0.05$ compared with 2-PAM group.

Figure 7. AChE activity in the red blood cell (RBC) and whole blood (WB) of the guinea pigs exposed to sarin (1.0 x LD_{50}, sc) and treated intramuscularly 5 min later with saline (as control), 58 µmol/kg of HLö7 and MMB-4, or 145 µmol/kg of 2-PAM, MINA and DAM. Blood was collected at 60 min after sarin exposure. AChE activities were expressed as mean % baseline activity $\pm$ SEM with N= 8 in each treatment group.
$^{\#}$ $p<0.05$ compared with agent/saline group (control).
$^{\circ}$ $p<0.05$ compared with HLö7 group.
$^{\square}$ $p<0.05$ compared with MMB-4 group.
* $p<0.05$ compared with 2-PAM group.

Figure 8. AChE activity in the red blood cell (RBC) and whole blood (WB) of the guinea pigs exposed to sarin (1.0 x LD_{50}, sc) and treated intramuscularly 5 min later with saline (as control), or various doses of MINA and DAM. Blood was collected at 60 min after sarin exposure. AChE activities were expressed as mean % baseline activity $\pm$ SEM with N= 8 in each treatment group.
$^{\#}$ $p<0.05$ compared with agent/saline group (control).
$^{\circ}$ $p<0.05$ compared with HLö7 group.
$^{\square}$ $p<0.05$ compared with MMB-4 group.
* $p<0.05$ compared with 2-PAM group.

Figure 9. An example of MMB-4 (26 mg/kg) treatment on sarin-induced EEG seizure activity. Animals were pretreated with pyridostigmine bromide 30 min prior to sarin (2 x LD_{50}) exposure and treated with MMB-4 and atropine sulfate (2.0 mg/kg) 1 min postexposure. (A) Baseline EEG activity. (B) Seizure onset at 6.5 min postexposure (arrow). (C-E) Seizure activity at 1, 4 and 24 hr, respectively, after onset.

Figure 10. An example of MINA (35 mg/kg) treatment on sarin-induced EEG seizure activity. Animals were pretreated with pyridostigmine bromide (0.026 mg/kg, im) 30 min prior to sarin (2 x LD_{50}, sc) exposure and treated intramuscularly with MINA and atropine sulfate (2.0 mg/kg, im) 1 min postexposure. (A) Baseline

EEG activity. (B) Seizure onset (arrow) at 3.9 min postexposure. (C) Peak seizure activity. (D) Seizure termination at 7.5 min after onset. (E and F) EEG recordings at 4 and 24 hr, respectively, show no signs of seizure activity.

Figure 11. An example of DAM (41 mg/kg) treatment on sarin-induced EEG seizure activity. Animals were pretreated with pyridostigmine bromide (0.026 mg/kg, im) 30 min prior to sarin (2 x LD_{50}, sc) exposure and treated intramuscularly with DAM and atropine sulphate (2.0 mg/kg) 1 min postexposure. (A) Baseline EEG activity. (B) Seizure onset (arrow) at 9.5 min postexposure. (C-E) Seizure activity at 1, 4 and 24 hr, respectively, after onset.

Figure 12. The 24-hr body weight loss in animals that survived a 2.0 x LD_{50} dose of sarin, with EEG seizure activity terminated (seizure off) or not terminated (seizure on) by tertiary or quaternary oxime treatments. All animals were pretreated with pyridostigmine bromide (0.026 mg/kg, im) at 30 min prior to sarin and treated intramuscularly with atropine sulfate (2.0 mg/kg) and an oxime at one min after sarin challenge. Body weight of each animal was recorded before sarin challenge and at 24 hr after sarin exposure in survivors.
$^{*}P<0.001$ compared with tertiary oximes seizure “off” group.

Figure 13. The dose-response curves for prevention/termination of seizures by MINA and DAM following sarin (2 x LD_{50}, sc) challenge in guinea pigs. All animals were pretreated with pyridostigmine bromide (0.026 mg/kg, im) at 30 min prior to sarin and treated intramuscularly with atropine sulfate ($ATSO_4$; 2.0 mg/kg) and an oxime at one min after sarin challenge.

Table 1: Toxic Sign Scores of Guinea Pigs Following Nerve Agent Exposure*

Toxic Signs	Score	Level/degree of toxicity (Total scores)
Salivation (1)	1	Mild (1-3)
Lacrimation (1)	1	
Nystagmus(1)	1	
General Motor (0-3)		Moderate (4-6)
Fasciculation	1	
Tremor	2	
Convulsion	3	
General State (0-3)		Severe (7-9)
Mild uncoordination	1	
Impaired movement	2	
Prostration	3	

* Guinea pigs were scored for absence [0] or presence of salivation [1], lacrimation [1], nystagmus [1], and the general motor deficits, which ranged from 1-3, for the presence of fasciculation [1], tremor [2], or convulsion [3]. Additionally, the guinea pig was allowed to walk on the bench top and the general state scores, which ranged from 0-3, were recorded with the following ranks: normal [0], mild uncoordination [1], impaired movement/with righting reflex [2], or prostration/without righting reflex [3]. A cumulative score was then calculated by tabulating the salivation, lacrimation, nystagmus, general motor and general state scores for each subject. The maximal attainable score was 9. A cumulative score was categorized as mild intoxication [1-3], moderate intoxication [4-6] or severe intoxication [7-9]. Modified for guinea pig from Shih and Romano (1988).

Table 2: Incidence and Severity of Toxic Sign Scores in Guinea Pigs Exposed to Sarin and Treated with Various Oximes*

Treatment (mg/kg)	# Animals Showing Signs**	Ave. Score
Saline	14/15	4.07
HLö7 (30.2)	8/8	4.13
MMB-4 (26)	7/7	3.43
2-PAM (25)	8/8	2.75
MINA (12.63)	0/7	0.00
MINA (17.5)	2/8	0.88
MINA (35)	2/8	0.25
MINA (60)	5/8	0.75
MINA (80)	1/8	0.13
DAM (14.66)	0/8	0.00
DAM (23)	1/8	0.13
DAM (41)	4/8	0.88
DAM (73)	3/8	0.38
DAM (128.8)	0/8	0.00

*Guinea pigs were injected subcutaneously with a 1.0 x LD_{50} dose (42 µg/kg) of sarin, followed 5 min later with intramuscular saline (as control) or a specified dose of an oxime. Toxic sign scores (based on Table 1) were taken at 58 min after sarin exposure for each animal and averaged for the same treatment group.

**Numbers of animals shown toxic signs/total numbers of animals used in each treatment group.

Table 3: AChE Activity in Brain Regions and Spinal Cord in Control and Sarin-Intoxicated Guinea Pigs*

Control AChE Activity (µmol substrate hydrolyzed/g protein/min),# mean ± SEM							
Tissue	Brainstem	Cerebellum	Cortex	Hippo	Midbrain	Spinal Cord	Striatum
Control	211.45 ±6.99	234.66 ±6.38	56.91 ±1.42	99.20 ±1.90	159.79 ±4.23	189.59 ±7.98	389.27 ±9.15
% of Control AChE Activity at 60 min,# mean ± SEM							
Sarin	19.35 ±3.58	10.68 ±2.13	10.26 ±1.50	23.71 ±3.06	13.90 ±1.91	29.53 ±3.57	19.80 ±11.84

*Guinea pigs were injected subcutaneously with saline (0.5 ml/kg, served as control) or a 1.0 x LD_{50} dose (42 µg/kg) of the nerve agent sarin. Brain regions and spinal cord were collected at 60 min after treatment.

#Control AChE activity was expressed as µmol substrate hydrolyzed/g protein/min. The AChE activity in sarin-exposed group was expressed as percentage of saline-treated control group. Values shown are mean ± S.E.M. with group size N= 37 for control and 9 for sarin, respectively.

Table 4: AChE Activity in Brain Regions of Guinea Pigs Exposed to Sarin (GB) Followed by Oxime Therapy

	% of Control AChE Activity[#]						
Treatment group	**Brainstem**	**Cerebellum**	**Cortex**	**Hippocampus**	**Midbrain**	**Spinal Cord**	**Striatum**
GB/saline	19.35	10.68	10.26	23.71	13.90	29.53	19.80
145 µmol/kg							
MINA (12.63 mg/kg)	39.05*	26.99	20.41	36.96	33.24*	48.62	47.46*
DAM (14.66 mg/kg)	37.47	27.61*	23.52	37.17	31.90*	50.75*	41.85*
2-PAM (25 mg/kg)	20.75	13.77	15.60	22.78	15.01	25.89	22.01
58 µmol/kg							
HLö7 (30.2 mg/kg)	16.55	11.77	13.42	27.00	14.52	28.09	20.17
MMB-4 (26 mg/kg)	21.67	13.29	13.10	30.38	15.81	32.87	28.46

AChE activity was expressed as % of baseline activity in each brain region. Guinea pigs were injected subcutaneously with a 1.0 x LD_{50} dose (42 µg/kg) of sarin (GB) and followed 5 min later with intramuscular saline (as control) or a specified dose of oxime. Brain regions and spinal cord were collected at 60 min after treatment. Group size N = 9 for GB/saline and 7-8 for oxime treatment groups.

* $p<0.05$ when compared with GB/saline control.

Table 5: AChE Activity in Peripheral Tissues and Blood in Control and Sarin-Intoxicated Guinea Pigs*

Control AChE Activity,# mean ± SEM					
	µmol/g protein/min			µmol/ml/min	
Treatment	Diaphragm	Heart	Skeletal Muscle	RBC	WB
Control	14.32±0.76	18.23±0.56	10.50±0.43	2.18±0.06	2.45±0.06
% of Control AChE Activity at 60 min,# mean ± SEM					
Sarin	27.84±3.53	17.40±2.87	40.39±5.67	6.65±2.16	8.52±0.73

*Guinea pigs were injected subcutaneously with saline (0.5 ml/kg) or a 1.0 x LD_{50} dose (42 µg/kg) of the nerve agent sarin. Peripheral tissues (diaphragm, heart and skeletal muscle) and blood (red blood cells [RBC] and whole blood [WB]) were collected at 60 min after treatment.

#Control AChE activity was expressed as µmol substrate hydrolyzed/g protein/min for peripheral tissue samples and as µmol substrate hydrolyzed/ml/min for blood samples. The AChE activity in sarin-exposed group was expressed as percentage of saline-treated control group. Values shown are mean ± S.E.M. with group size N= 37 for control and N= 9 for sarin, respectively.

Table 6: AChE Activity in Peripheral Tissues and Blood of Guinea Pigs Exposed to Sarin (GB) Followed by Oxime Therapy

	% of Control AChE Activity[#]				
Treatment group	**Diaphragm**	**Heart**	**Skeletal**	**RBC**	**WB**
GB/saline	27.84	17.40	40.39	6.65	8.52
145 µmol/kg					
MINA (12.63 mg/kg)	32.44	16.08	41.82	5.14	11.10
DAM (14.66 mg/kg)	32.65	16.19	43.03	4.94	8.58
2-PAM (25 mg/kg)	62.48*	61.70*	70.23*	52.52*	61.78*
58 µmol/kg					
HLö7 (30.2 mg/kg)	80.10*	58.68*	104.78*	52.15*	66.78*
MMB-4 (26 mg/kg)	90.09*	56.19*	88.73*	75.34*	83.13*

AChE activity was expressed as % of baseline activity in each peripheral tissue (diaphragm, heart and skeletal muscle) or blood (red blood cells [RBC] and whole blood [WB]). Guinea pigs were injected subcutaneously with a 1.0 x LD_{50} dose (42 µg/kg) of sarin (GB) and followed 5 min later with intramuscular saline (as control) or a specified dose of oxime. Peripheral tissues and blood (RBC and WB) were collected at 60 min after treatment. Group size N = 9 for GB/saline and 7-8 for oxime treatment groups.

* $p<0.05$ when compared with GB/saline control.

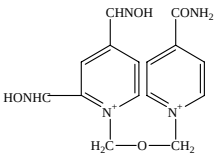
Table 7: Effects of Oxime on Sarin-Induced Seizure Occurrence, Termination and Survival

Treatment (mg/kg)	Never Seized	Seizure "OFF"	Survival (24 hr)
2-PAM (25)	0/10 (0%)	0/10 (0%)	5/10 (50%)
MMB-4 (26)	0/5 (0%)	0/5 (0%)	2/5 (40%)
HLö7 (30.2)	0/5 (0%)	0/5 (0%)	1/5 (20%)
MINA (20)	0/7 (0%)	0/7 (0%)	3/7 (43%)
(26)	1/11 (9%)	1/11(9%)	7/11 (64%)
(35)	2/12 (17%)	7/12 (58%)	9/12 (75%)
(46)	6/10 (60%)	9/10 (90%)	9/10 (90%)
(60)	9/12 (75%)	11/12 (92%)	12/12 (100%)
DAM (41)	0/7 (0%)	0/7 (0%)	5/7 (71%)
(73)	1/6 (17%)	1/6 (17%)	5/6 (83%)
(129)	4/6 (67%)	4/6 (67%)	6/6 (100%)
(231)	6/6 (100%)	6/6 (100%)	6/6 (100%)

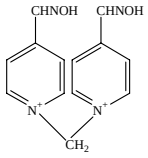
* Guinea pigs were pretreated with pyridostigmine bromide (0.026 mg/kg, im) 30 min prior to sarin (2 x LD_{50}, sc) and followed 1 min later by atropine sulfate (2.0 mg/kg, im) and a dose of an oxime (im). EEG seizure onset, termination and 24-hr survival were recorded (numbers of animal responded/total numbers of animals). Numbers of animals responded in Seizure “OFF” column included both animals that never seized and animals that seized but seizure activity was terminated soon after seizure onset.

Figure 1: Chemical Structures of Oximes

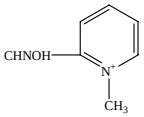
Quarternary Oximes



HLo7

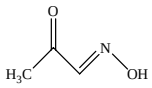


MMB-4

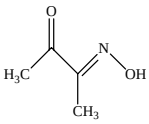


2-PAM

Tertiary Oximes



MINA



DAM

Figure 1. Chemical structures of quaternary and tertiary oximes studied.

Figure 2: Toxic Sign Scores

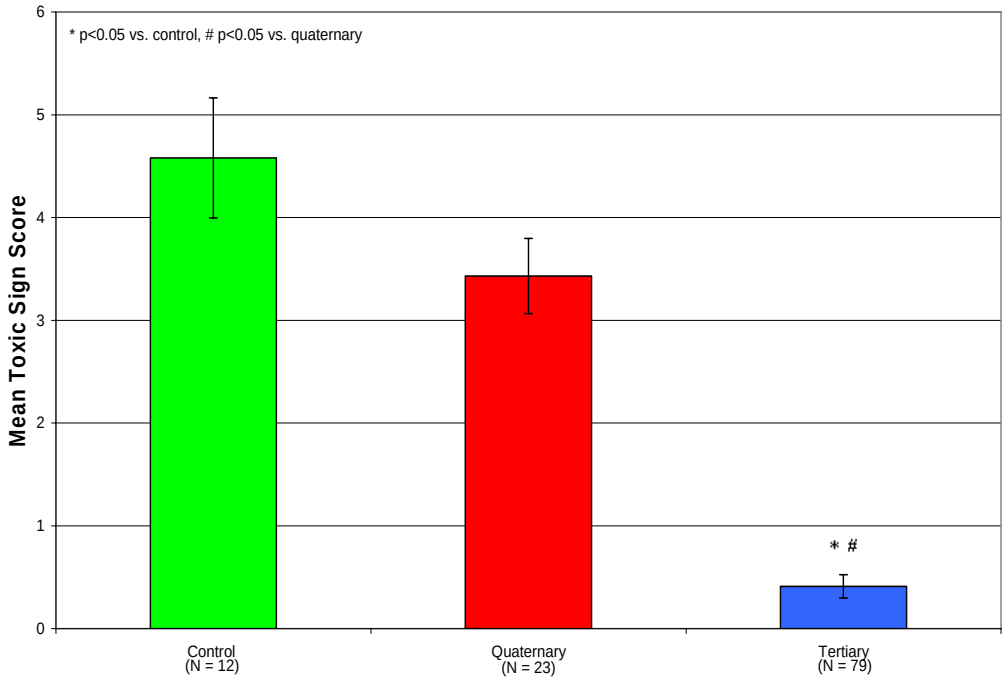


Figure 2. The overall toxic sign scores in animals exposed to a 1.0 x LD_{50} dose of sarin and treated 5 min later with saline (served as control), quaternary (2-PAM, MMB-4 and HLö7) or tertiary (DAM and MINA) oximes. Toxic signs were scored at 58 min after sarin administration in survivors.
[*]P<0.05 compared with saline control group.
[#] P<0.05 compared with quaternary oxime group.

Figure 3: AChE Activity in Striatum and Midbrain of Guinea Pigs: Equimolar Doses

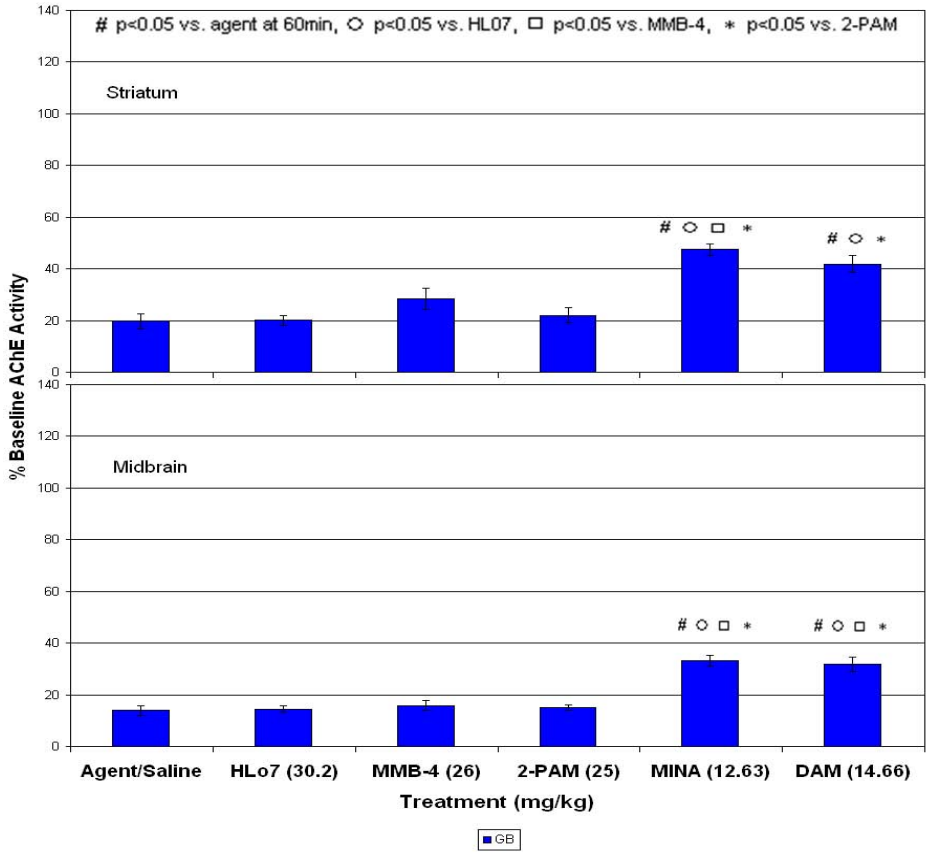


Figure 3. AChE activity in striatum and midbrain regions of the guinea pigs exposed to sarin (1.0 x LD_{50}, sc) and treated intramuscularly 5 min later with saline (as control), 58 µmol/kg of HLö7 and MMB-4, or 145 µmol/kg of 2-PAM, MINA and DAM. Brain tissues were collected at 60 min after sarin exposure. AChE activities were expressed as mean % baseline activity ± SEM with N= 8 in each treatment group.

[#] p<0.05 compared with agent/saline group (control).

[○] p<0.05 compared with HLö7 group.

[□] p<0.05 compared with MMB-4 group.

[*] p<0.05 compared with 2-PAM group.

Figure 4: AChE Activity in Striatum and Midbrain of Guinea Pigs: Dose-Respose

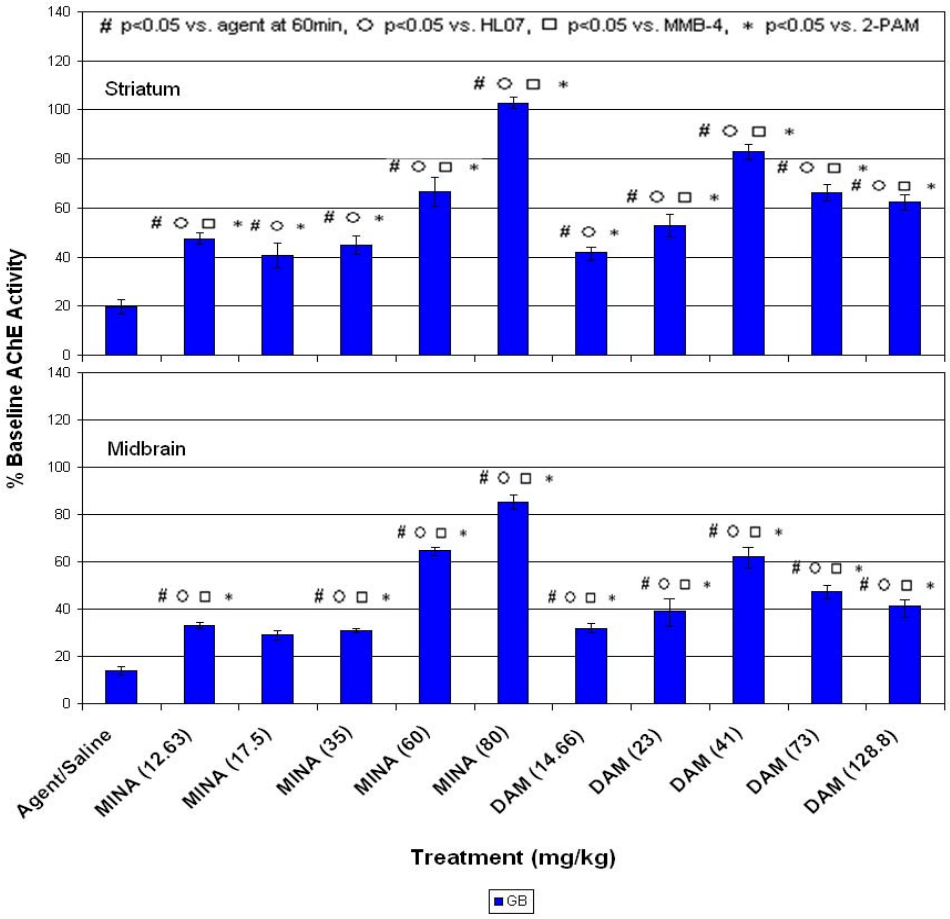


Figure 4. AChE activity in striatum and midbrain regions of the guinea pigs exposed to sarin (1.0 x LD_{50}, sc) and treated intramuscularly 5 min later with saline (as control) or various doses of MINA or DAM. Brain tissues were collected at 60 min after sarin exposure. AChE activities were expressed as mean % baseline activity $\pm$ SEM with N= 8 in each treatment group.

[#] p<0.05 compared with agent/saline group (control).

[○] p<0.05 compared with HLö7 group.

[□] p<0.05 compared with MMB-4 group.

[*] p<0.05 compared with 2-PAM group.

Figure 5: AChE Activity in Diaphragm, Heart and Skeletal Muscle of Guinea Pigs: Equimolar Doses

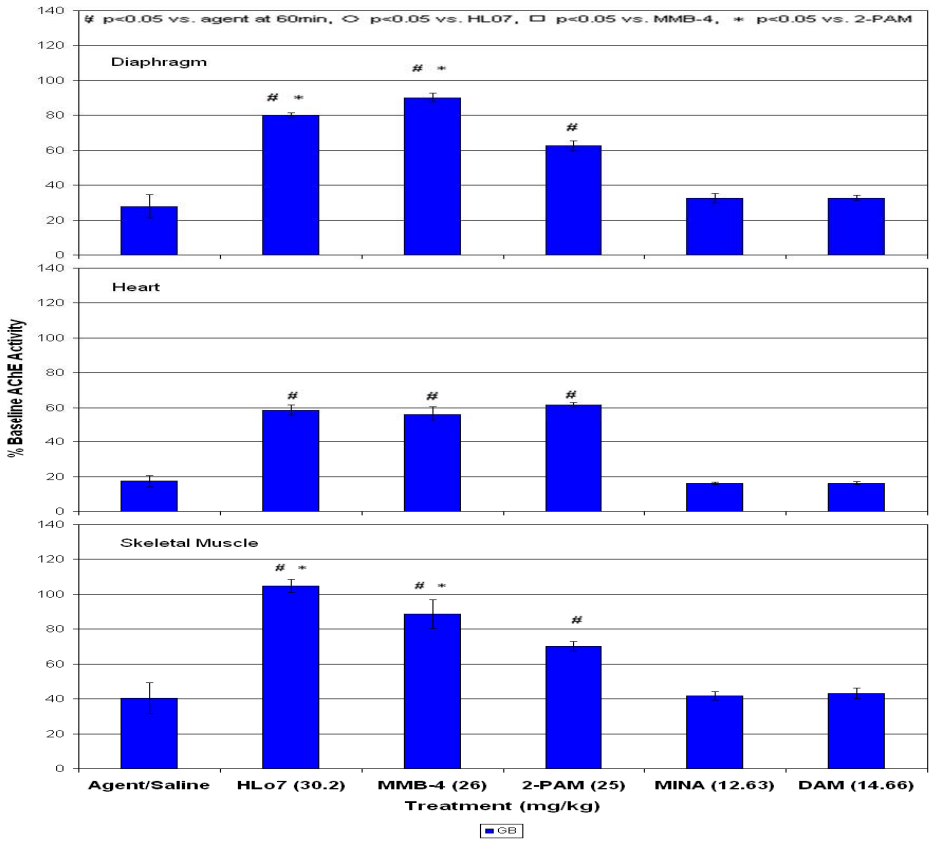


Figure 5. AChE activity in the diaphragm, heart and skeletal muscle of the guinea pigs exposed to sarin (1.0 x LD_{50}, sc) and treated intramuscularly 5 min later with saline (as control), 58 µmol/kg of HLö7 and MMB-4, or 145 µmol/kg of 2-PAM, MINA and DAM. Tissues were collected at 60 min after sarin exposure. AChE activities were expressed as mean % baseline activity ± SEM with N= 8 in each treatment group.

[#] p<0.05 compared with agent/saline group (control).
[○] p<0.05 compared with HLö7 group.
[□] p<0.05 compared with MMB-4 group.
[*] p<0.05 compared with 2-PAM group.

Figure 6: AChE Activity in Diaphragm, Heart and Skeletal Muscle of Guinea Pigs: Dose-Respose

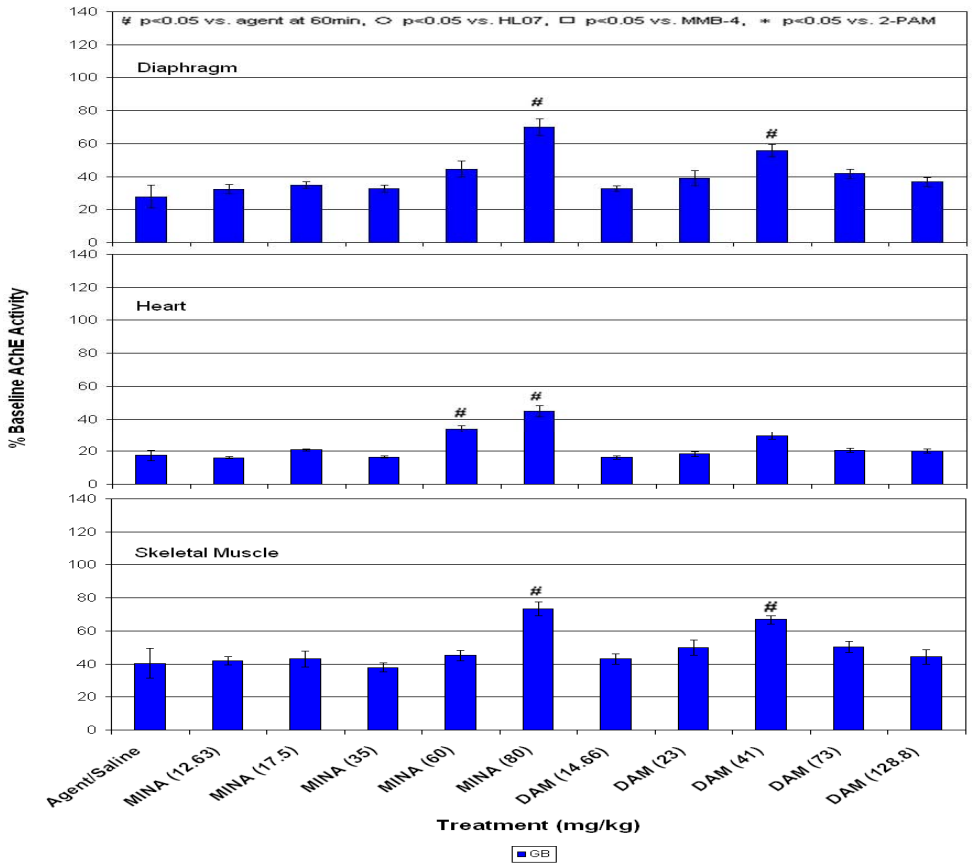


Figure 6. AChE activity in the diaphragm, heart and skeletal muscle of the guinea pigs exposed to sarin (1.0 x LD_{50}, sc) and treated intramuscularly 5 min later with saline (as control), or various doses of MINA or DAM. Tissues were collected at 60 min after sarin exposure. AChE activities were expressed as mean % baseline activity $\pm$ SEM with N= 8 in each treatment group.

[#] p<0.05 compared with agent/saline group (control).

[○] p<0.05 compared with HLö7 group.

[□] p<0.05 compared with MMB-4 group.

[*] p<0.05 compared with 2-PAM group.

Figure 7: AChE Activity in the Blood of Guinea Pigs: Equimolar Doses

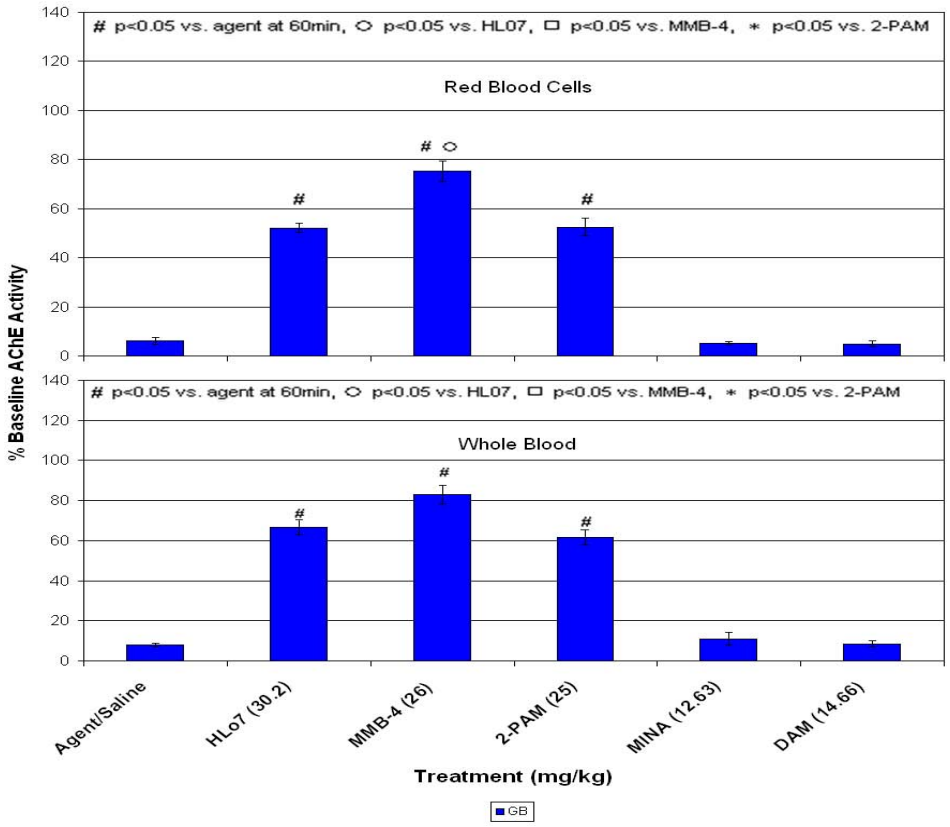


Figure 7. AChE activity in the red blood cell (RBC) and whole blood (WB) of the guinea pigs exposed to sarin (1.0 x LD_{50}, sc) and treated intramuscularly 5 min later with saline (as control), 58 µmol/kg of HLö7 and MMB-4, or 145 µmol/kg of 2-PAM, MINA and DAM. Blood was collected at 60 min after sarin exposure. AChE activities were expressed as mean % baseline activity ± SEM with N= 8 in each treatment group.

[#] p<0.05 compared with agent/saline group (control).

[○] p<0.05 compared with HLö7 group.

[□] p<0.05 compared with MMB-4 group.

[*] p<0.05 compared with 2-PAM group.

Figure 8: AChE Activity in the Blood of Guinea Pigs: Equimolar Doses

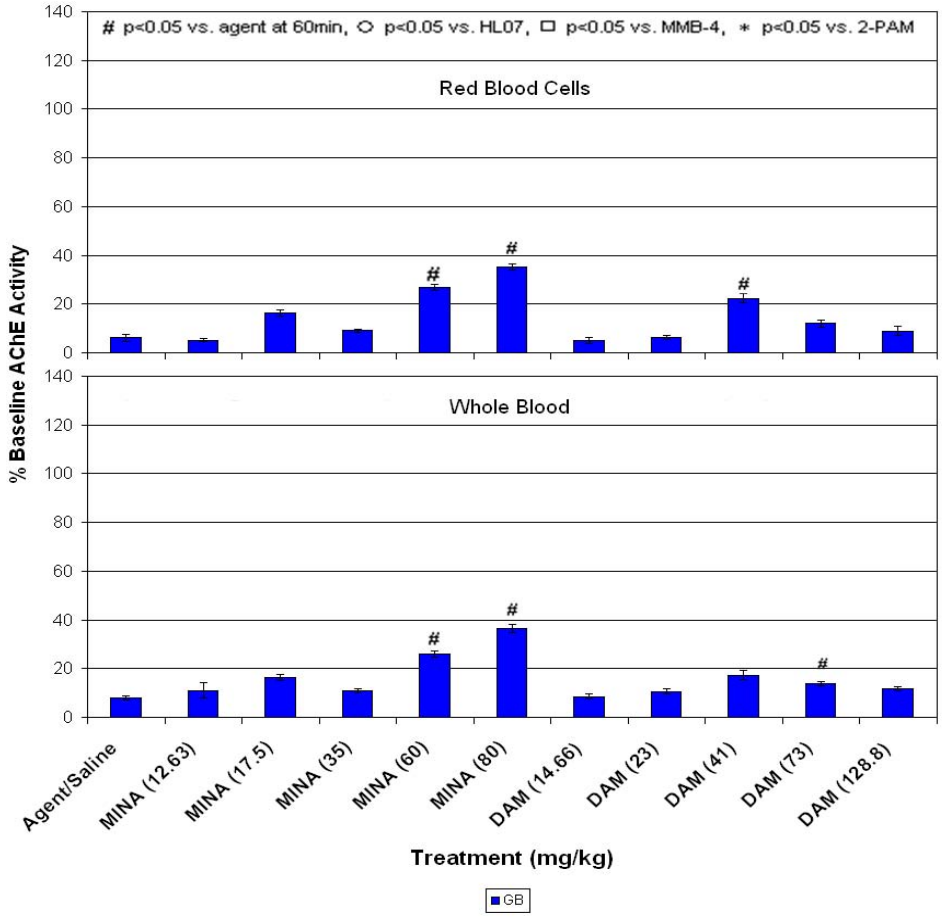


Figure 8. AChE activity in the red blood cell (RBC) and whole blood (WB) of the guinea pigs exposed to sarin (1.0 x LD_{50}, sc) and treated intramuscularly 5 min later with saline (as control), or various doses of MINA and DAM. Blood was collected at 60 min after sarin exposure. AChE activities were expressed as mean % baseline activity ± SEM with N= 8 in each treatment group.
[#] p<0.05 compared with agent/saline group (control).
[○] p<0.05 compared with HLö7 group.
[□] p<0.05 compared with MMB-4 group.
[*] p<0.05 compared with 2-PAM group.

Figure 9: EEG Tracings of Guinea Pig Treated with MMB-4

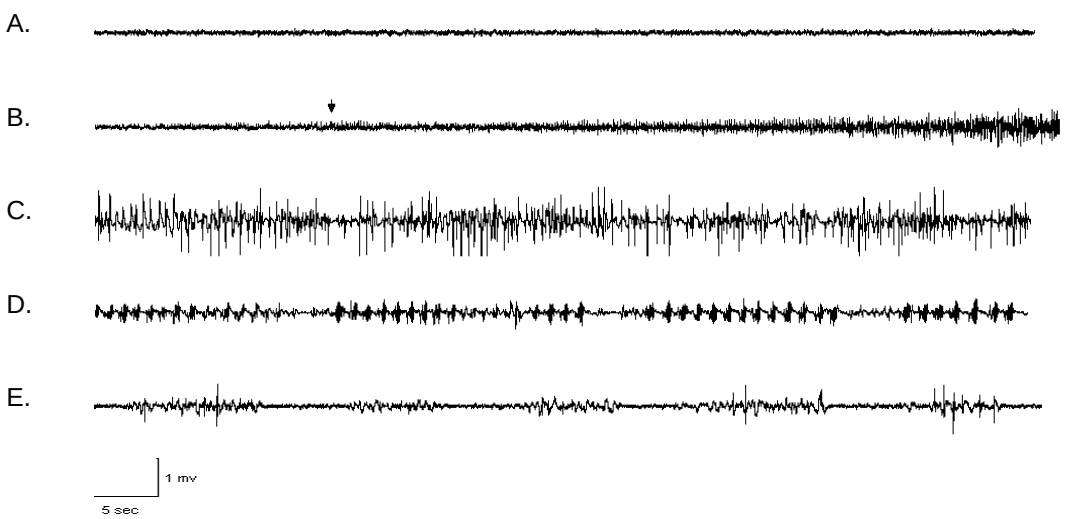


Figure 9. An example of MMB-4 (26 mg/kg) treatment on sarin-induced EEG seizure activity. Animals were pretreated with pyridostigmine bromide 30 min prior to sarin (2 x LD_{50}) exposure and treated with MMB-4 and atropine sulfate (2.0 mg/kg) 1 min postexposure. (A) Baseline EEG activity. (B) Seizure onset at 6.5 min postexposure (arrow). (C-E) Seizure activity at 1, 4 and 24 hr, respectively, after onset.

Figure 10: EEG Tracings of Guinea Pig Treated with MINA

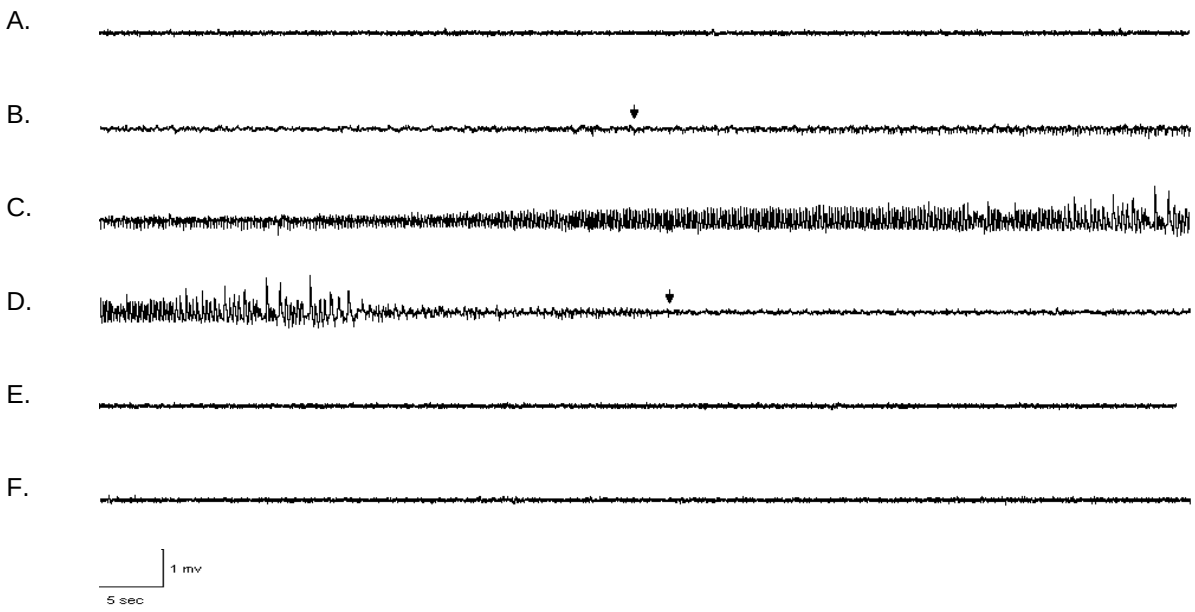


Figure 10. An example of MINA (35 mg/kg) treatment on sarin-induced EEG seizure activity. Animals were pretreated with pyridostigmine bromide (0.026 mg/kg, im) 30 min prior to sarin (2 x LD_{50}, sc) exposure and treated intramuscularly with MINA and atropine sulfate (2.0 mg/kg, im) 1 min postexposure. (A) Baseline EEG activity. (B) Seizure onset (arrow) at 3.9 min postexposure. (C) Peak seizure activity. (D) Seizure termination at 7.5 min after onset. (E and F) EEG recordings at 4 and 24 hr, respectively, show no signs of seizure activity.

Figure 11: EEG Tracings of Guinea Pig Treated with DAM

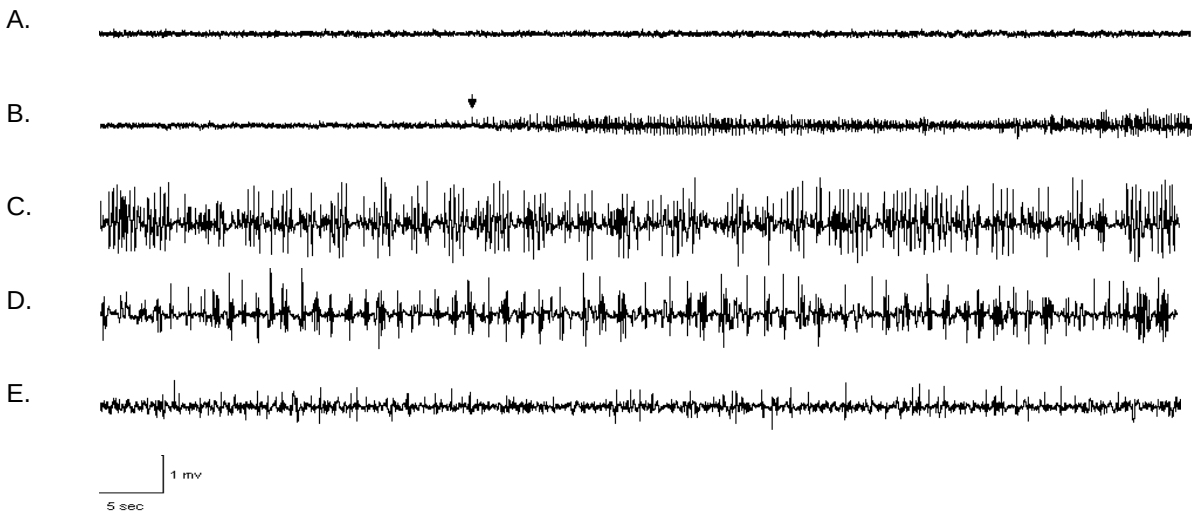
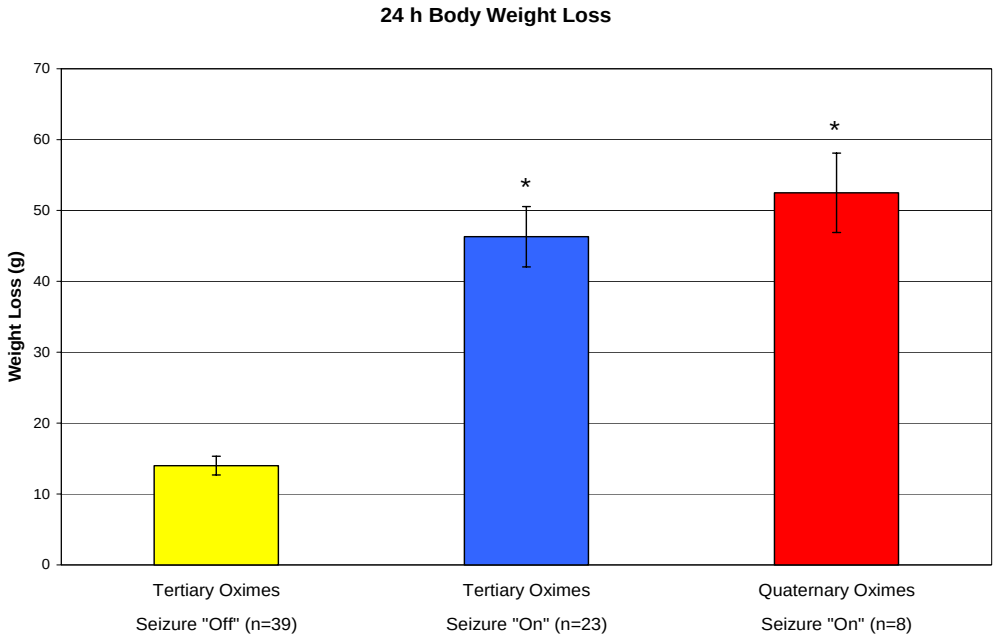


Figure 11. An example of DAM (41 mg/kg) treatment on sarin-induced EEG seizure activity. Animals were pretreated with pyridostigmine bromide (0.026 mg/kg, im) 30 min prior to sarin (2 x LD_{50}, sc) exposure and treated intramuscularly with DAM and atropine sulphate (2.0 mg/kg) 1 min postexposure. (A) Baseline EEG activity. (B) Seizure onset (arrow) at 9.5 min postexposure. (C-E) Seizure activity at 1, 4 and 24 hr, respectively, after onset.

Figure 12: Body Weight Loss in 24 Hours

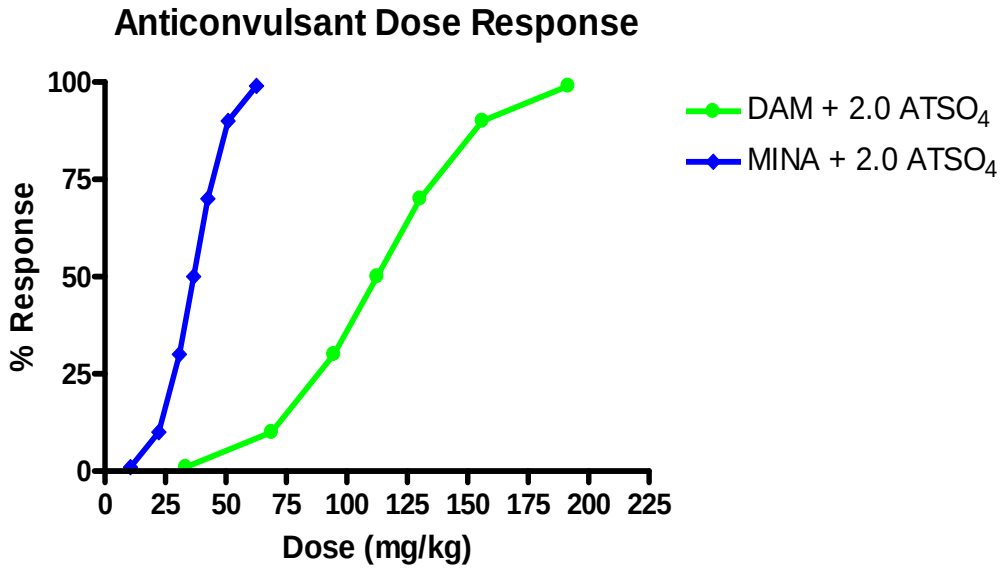


	Seizure "Off" (n=39) Tertiary Oximes	Seizure "On" (n=23) Tertiary Oximes	Seizure "On" (n=8) Quaternary Oximes
mean	14	46.3	52.5
N	39	23	8
S.D.	8.2	20.4	15.9
SEM	1.3	4.2	5.6

Figure 12. The 24-hr body weight loss in animals that survived a 2.0 x LD_{50} dose of sarin, with EEG seizure activity terminated (seizure off) or not terminated (seizure on) by tertiary or quaternary oxime treatments. All animals were pretreated with pyridostigmine bromide (0.026 mg/kg, im) at 30 min prior to sarin and treated intramuscularly with atropine sulfate (2.0 mg/kg) and an oxime at one min after sarin challenge. Body weight of each animal was recorded before sarin challenge and at 24 hr after sarin exposure in survivors.

*$P<0.001$ compared with tertiary oximes seizure “off” group.

Figure 13: Anticonvulsant Dose-Response Curves for DAM and MINA



Summary Of Anticonvulsant ED_{50} Doses

Treatments	Dose (mg/kg)
MINA + $ATSO_4$ (2.0 mg/kg)	36.65 (0.00 - 76.30)
DAM + $ATSO_4$ (2.0 mg/kg)	112.51 (83.78 - 178.87)

Figure 13. The dose-response curves for prevention/termination of seizures by MINA and DAM following sarin (2 x LD_{50}, sc) challenge in guinea pigs. All animals were pretreated with pyridostigmine bromide (0.026 mg/kg, im) at 30 min prior to and treated intramuscularly with atropine sulfate ($ATSO_4$; 2.0 mg/kg) and an oxime at one min after sarin challenge.

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